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(57) Abstract

A transformant selection system has been developed, particularly for a β-lactam producing strain, more particularly for *P. chrysogenum*, by the complementation of a mutation of said β-lactam producing strain by a homologous selection marker without interfering with β-lactam biosynthesis. Particularly, in applying said transformant selection system a positive selection agent, for instance fluoroacetate is used for the isolation of *fac* mutants of said strain, particularly of said β-lactam producing strain. Furthermore, a gene entitled *facA* has been isolated from *P. chrysogenum*.

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Transformation selection marker system adapted for penicillium.

The present invention relates to a transformant selection system, particularly for a strain of fungus, more particularly Penicillium chrysogenum.

Furthermore, this invention relates to a transformant selection marker, viz. the acetyl-CoA synthetase (facA) gene, isolated from Penicillium chrysogenum.

The filamentous fungus Penicillium chrysogenum is the most applied producer of the β-lactam compounds penicillin G and penicillin V. Penicillins G/V are used as antibiotics themselves or they are chemically converted into semi-synthetic β-lactams. P. chrysogenum has a long record of industrial application. Since the second world war it has been the microorganism of choice for large scale production of penicillins all over the world. Over the years significant improvements have been made in the yield of the penicillin production process, both by strain improvement and by process development. Strain improvement has been pursued by the application of random mutagenesis by chemical and physical means as well as by targeted mutagenesis of key enzymes in metabolic pathways connected to penicillin biosynthesis, followed by extensive selections for strains with increased penicillin titers. For reviews see for example Hersbach, G.J.M., Van der Beek, C.P. and Van Dijck, P.W.M., "The Penicillins: Properties, Biosynthesis and Fermentation" in Biotechnology of Industrial Antibiotics, E. van Damme (ed), Marcel Dekker (NY), 1984 and Rowlands, R.T., Enzyme Microb. Technol. 6, 1984, 3-10 and 290-300.

A novel approach to strain improvement has become possible with the development of recombinant DNA technology. Prerequisites for the application of recombinant DNA techniques to any organism or cell-line, ar the availability of a gene transfer and a selection or detection system, which permits the identification of the usually small number of recombinant DNA contain-

ing transformed cells, in a vast majority of non-transformed cells. The development of efficient gene transfer and selection systems for β-lactam producing industrial strains, particularly for P. chrysogenum is very difficult for two major reasons. First of all, it is a common observation that β-lactam producing industrial strains are by far more difficult to transform than wild type or laboratory strains (Ingolia & Queener, Med. Res. Rev. 9, 1989, 245-264). The feature of impaired transformation of industrial strains might be related to the extensive mutagenesis of these strains. For example, mutations affecting the composition and/or assembly of the cell membrane or the cell wall are likely to accumulate in industrial strains leading to changes in morphology in the course of a strain improvement program. (Lein, in: Overproduction of Microbial Metabolites, Vanek and Hostálek (eds), 1986, Butterworth Publishers, 105-139). These morphological changes might for instance affect the generation of protoplasts from mycelium, the stability of protoplasts, their capacity for uptake of DNA, the regeneration of protoplasts into mycelium etc. Secondly, it is an additional requirement that the gene transfer and selection procedure should not affect the level of penicillin production.

The difficulties encountered in the development of transformant selection systems are also related to the limited knowledge of the genetic system of P. chrysogenum, which is difficult to study (e.g. because of the multinucleate nature of the filamentous mycelium and the absence of a sexual cycle, which only permits parasexual analysis (Pontecorvo et al., Adv. Genet. 5, 1953, 141-238)), the physical barriers which hinder the uptake of exogenous DNA (Peberdy, in: Biochemistry of Cell Walls and Membranes in Fungi; Kuhn, P.J., Trinci, A.P.J., Jung, M.J., Gossey, M.W., Copping, L.G. (eds) Springer-Verlag, Berlin 1989, 5-30) and the lack of DNA elements which allow for stable extrachromosomal replication of the transforming DNA, which consequently results in very low transformation fr qu ncies because the transforming DNA has to integrate into the genome of the host.

At this moment several systems for the selection of transformants have been described for P. chrysogenum. However, although the development of these selection systems has been useful in itself from a scientific point of view, the selection systems in current use suffer each from one or several of the following drawbacks which hinder their application to β-lactam producing industrial strains, particularly of P. chrysogenum.

Firstly, in some selection systems the phenotype selected for is conferred to P. chrysogenum by heterologous DNA (EP-A-240250; EP-A-215539; EP-A-225078; Cantoral et al., Bio/technology 5, 1987, 494-497; Beri and Turner, Curr. Genet. 11, 1987, 639-641; Kolar et al., Gene 62, 1988, 127-134; Stahl et al., App. Microbiol. Biotechnol. 26, 1987, 237-241; Picknett and Saunders, FEMS Microbiol. Lett. 60, 1989, 165-168; Whitehead et al., Mol. Gen. Genet. 216, 1989, 408-411). As a consequence of the public concern on recombinant DNA technology in general, the use of a transformant selection system for a β-lactam producing strain based upon a selection marker which consists of homologous DNA rather than heterologous DNA is preferred. Furthermore, from a practical point of view, transformation frequencies are usually higher when using homologous rather than heterologous selection markers.

Secondly, some selection systems depend on the generation of auxotrophic mutants of P. chrysogenum (EP-A-235951; EP-A-260762; Picknett et al., Curr. Genet. 12, 1987, 449-445; Diez et al., Curr. Genet. 12, 1987, 277-282). Generally spoken, the isolation of specific auxotrophic mutants requires extensive identification of mutant strains and is therefore rather time-consuming. This is a serious disadvantage when different hosts are used (e.g. in industrial strain improvement programs). In addition, and even more importantly, the introduction of auxotrophic mutations in industrial strains of P. chrysogenum often results in an unacceptable reduction of biosynthesis of penicillin. This phenomenon may be a consequence of the mutagenic treatment, necessary to introduce the required auxotrophic mutations in the strain of interest or may be related to particular auxotrophic defects per se (see for example 'O Sullivan

and Pirt, J. Gen. Microbiol. 76, 1973, 65-75 and Stahl et al., ibid).

In P. chrysogenum breeding programs, aimed at the development of strains with increased penicillin production levels, the introduction of genetic markers, which require mutagenesis is therefore usually avoided (see for example Bein, In: Overproduction of Microbial Metabolites, Vanek, Z., Hostálek, Z. (eds), Butterworths, Boston, 1987, 105-140).

In conclusion, homologous selection systems which do not require mutagenesis of the host, like dominant selection systems or selection systems employing hosts which can be obtained by positive selection for a spontaneous mutation, are highly preferred. An example of the first category is the semi-dominant, homologous oliC selection system (EP-A-311272). However, a serious disadvantage of the oliC selection system is the very low frequency of transformation which limits the application of this selection system. An example of the second category is the niaD selection system which employs niaD mutants of Penicillium chrysogenum, obtained by positive selection for resistance to chlorate (Whitehead et al., ibid., AT patent application 8900266). Since resistance to chlorate can be obtained by spontaneous mutations at many different loci extra growth tests are necessary to identify the niaD mutants which form a drawback of this system. Another disadvantage of the niaD selection system is the observation that a large proportion of the P. chrysogenum transformants are genetically unstable (abortives), see Gouka et al., J. Biotechn. 20, 1991, 189-200.

Thirdly, for the application of recombinant DNA techniques in an industrial strain improvement program of β-lactam producing strains it is very important that a strain which has been transformed once can easily be transformed for a second time. Successive transformations have not shown to be possible in an efficient manner using the current selection systems. This feature is relevant as well for scientific studi s on regulation of gene expression in P. chrysogenum and other filamentous fungi.

In summary, a convenient and reusable transformant sel c-tion system for P. chrysogenum based on the use of a homologous selection marker, suitable for application to industrial strains of a β-lactam producing micro-organism, particularly of P. chrysogenum is not available.

A system for the selection of transformants, particularly of a fungus, more particularly of a β-lactam producing strain of fungus, most particularly of P. chrysogenum, has now been developed which lacks the drawbacks of current selection systems. This selection system is based upon the complementation of fac, preferably facA mutants of β-lactam producing strains, particularly of P. chrysogenum (fac stands for fluoroacetate resistant) by transformation with the P. chrysogenum facA gene, encoding acetyl-CoA synthetase.

Recently, the facA gene of A. nidulans and the corresponding acu-5 gene of N. crassa have been isolated and characterized by nucleotide sequence analysis (Sandeman and Hynes, Mol. Gen. Genet. 218, 1989, 87-92; Thomas et al., Molec. Microbiol. 2, 1988, 599-606; Connerton et al., Molec. Microbiol. 4, 1990, 451-460). The facA gene of the corn smut pathogen Ustilago maydis has also been isolated (Hargreaves and Turner, J. Gen. Microbiol. 135, 1989, 2675-2678). Fac mutants are phenotypically characterized by their inability to grow on acetate as a sole source of carbon. Therefore, Fac^+ transformants should be selectable for their regained capacity for acetate-utilization. However, the development of an efficient direct transformant selection system based on acetate-utilization appears to be difficult for A. nidulans and N. crassa (Ballance and Turner, Mol. Gen. Genet. 202, 1986, 271-275; Connerton et al, ibid) and U. maydis (Hargreaves and Turner, ibid).

The facA mutation can be selected for in P. chrysogenum with no need for mutagenic treatments like UV (ultra-violet) irradiation, exposure to chemical mutagens and the like. Spontaneous facA mutants, among others, can b isolated surprisingly efficiently by using a positive s lection for these mutants which are resistant to fluoroacetate. Stable facA mutants with very low reversion frequencies are readily obtained.

In Aspergillus nidulans and Coprinus lagopus mutations at three distinct genetic loci, termed facA, facB, facC (Apirion, Nature 195, 1962, 959-961) and acu-1, acu-11, acu-12 (Casselton and Casselton, Mol. Gen. Genet. 132, 1974, 255-264) respectively, have been identified, each of them resulting in a fluoroacetate resistant, acetate non-utilizing phenotype. In addition to these fac/acu mutants, the same studies on A. nidulans and C. lagopus describe the isolation of a large number of fluoroacetate resistant but acetate-utilizing mutants, which are designated fanA, fanB, fanC, fanD and FanE in A. nidulans (Apirion, ibid). In contrast to this complex set of mutants the selection of facA mutants of P. chrysogenum is surprisingly efficient. Nearly all of the fluoroacetate resistant mutants of P. chrysogenum are acetate non-utilizers, mutated at the facA locus. A high proportion of these P. chrysogenum facA mutants have unaltered penicillin production characteristics as compared to the parent strain.

In the present invention a method for the efficient, direct selection of FacA$^+$ transformants of a β-lactam producing strain, particularly of P. chrysogenum on acetate containing medium is established by using the facA gene of P. chrysogenum as a homologous selection marker.

A homologous selection marker is defined in the present patent application as a selection marker derived from the species to which the transformant selection system is applied.

By the invention also the facA gene particularly isolated from Penicillium chrysogenum has been provided for. The invention also includes genes comprising different nucleotide sequences for instance with conservative mutations, where the sequence encodes the same amino acid sequence, but may have as many as 15% different bases, or mutations which are non-conservative, where fewer than about 10%, more usually fewer than about 5%, and preferably not more than 1% of the amino acids are substituted or deleted, and there are fewer than 5% of inserted amino acids, where the percent is based on the number of naturally occurring amino acids.

An additional advantage of the facA selection system over other selection systems is the rapid sporulation of acetate-utilizing colonies on acetate containing selection medium, which greatly improves and accelerates the transformant selection procedure. FacA$^+$ transformants are stable because the transforming DNA is integrated into the genome. The facA transformant selection system is suitable for the generation of different types of transformants, containing the transforming DNA integrated at the resident facA locus, at unknown genomic sites, in single and/or multiple copies. The generation of single-copy FacA$^+$ transformants by using circular, double-stranded DNA is surprisingly efficient. This feature allows for rapid isolation of facA mutants of the FacA$^+$ transformant and a repetition of the facA transformant selection procedure. This is another great advantage of the facA selection system.

The facA selection system can be applied to introduce non-selectable DNA into a host, preferably P. chrysogenum. The non-selectable DNA can be used to obtain or to enhance the production of a β-lactam compound in a host, preferably P. chrysogenum, for example by using non-selectable DNA like penicillin, cephalosporin or cephamycine biosynthetic genes (Veenstra et al., J. Biotechn. 17, 1991, 81-90, and Cantwell et al., Curr. Genet. 17, 1990, 213-221).

This invention provides a method to select transformants of a microorganism which has been transformed with DNA which method comprises:

isolating a mutant of the microorganism in which acetyl-CoA synthetase is inoperable or absent;

cotransforming said mutant with said DNA and an expression system effective in producing acetyl-CoA synthetase of Penicillium chrysogenum; and

selecting transformants of said microorganism for ability to grow on a medium which contains a carbon source which requires acetyl-CoA synth tase activity for catabolism.

Furthermore, the invention provides a method for obtaining or enhancing the production of a β-lactam compound in transformants of a microorganism by applying th abov -mentioned

selection method which method comprises cotransforming said mutant with DNA encoding gen tic information necessary for obtaining or enhancing the production of a β-lactam compound.

Preferably, the above-mentioned methods are applied homologously by using Penicillium chrysogenum transformed with the P. chrysogenum facA gene.

Finally the invention provides a gene entitled facA having the nucleotide sequence depicted in Sequence listing 1, particularly isolated from P. chrysogenum, and a vector and a host comprising the same. Also the facA gene wherein one or both of the expression signals has been replaced by other expression signals, obtained from the same or another organism has been provided together with a vector and a host comprising the same.

Brief description of the figures

Figure 1: Schematic representation of the P. chrysogenum DNA contained in lambda EMBL-3 phage facA7. The position and the direction of transcription of the facA gene is indicated (arrow). E:EcoRI, P:PstI, S:SalI.

Figure 2: Southern blot analysis of PstI digested genomic DNA isolated from P. chrysogenum FacA$^+$ transformants by hybridization with (A) the 6.5 kb PstI fragment containing the facA gene of P. chrysogenum and (B) plasmid pPC1-1, for the detection of non-P. chrysogenum vector sequences in the transformants. The hybridizing band of approximately 7 kb in panel B is derived from hybridization of niaD sequences contained in pPC1-1 with the corresponding chromosomal niaD sequences. The intensity of this hybridization signal has been used as an internal standard for the amount of DNA loaded. The position of DNA size-markers is indicated p: pPC2-3; wt: P. chrysogenum; 1-14: different FacA$^+$ transformants.

Brief description of the sequenc listings

Sequence listing 1: Nucleotide sequence of the facA gene of P.chrysogenum and derived sequence of amino acids.

Sequence listing 2: Amino acid sequence of acetyl CoA synthetase of P. chrysogenum.

Transformation of industrial strains of Penicillium chrysogenum by recombinant DNA can be achieved using methods well-known in the art (Peberdy, Mycol. Res. 93, 1989, 1-20). In a preferred embodiment of the invention, mycelium is harvested from a fresh culture and protoplasts are generated from the filamentous mycelium by enzymatic treatment, i.e. by Novozyme 234, in an osmotically stabilized medium. Then, DNA and protoplasts are mixed together in a Ca^{2+} containing solution. Usually, several µg of DNA are added to 10^7-10^8 protoplasts. Subsequently, polyethyleneglycol (PEG) is added to the mixture to mediate DNA uptake by the protoplasts. Finally, the protoplasts are plated onto an osmotically stabilized selection medium. Other techniques for the delivery of DNA into target cells have been described, i.e. transformation by electroporation (Richey et al., Phytopathology 79, 1989, 844-847), by biolistic™ (Du Pont Particle Delivery System) methods (Armaleo et al., Curr. Genet. 17, 1990, 97-103) or by liposome delivery systems (Felger and Holm, Focus 11, 1989, 21-25). The application of these techniques to filamentous fungi is still in its infancy, but in the scope of the invention the application of any chemical, physical or biological method for transformation of P. chrysogenum is envisaged.

Typical results obtained using the selection system described herein, are: 1) transformation frequencies, in the order of 1-100 transformants per µg of DNA, and 2) the observation that in all stable transformants the recombinant DNA is integrated into the genome. These results are typical for transformation systems of filamentous fungi (Rambosek and Leach, Critical Rev. Biotechn. 6, 1987, 357-393; Timberlake and Marshall, Science 244, 1989, 1313-1317; Peberdy, Mycol. Res. 93, 1989, 1-20).

As concerns the frequency of transformation, the possibility exists that the efficiency of the facA transformation procedure can be increased further by systematic variation of the reaction parameters in the transformation procedure, as has been described for example by Picknett for the trpC selection procedure (Picknett, British Thesis, DX 82490, British Library, Document Supply Centre, Boston Spa, Wetherby, UK). As concerns the nature of the integration event in the transformation process, it has been observed that transformation with double-stranded circular DNA results in three different types of integration of the vector: integration into unknown genomic sites ("type II" integration), into the resident facA locus ("type I" integration) and by gene-conversion or gene-replacement of the mutant allele ("type III" integration) (Rambosek and Leach, Critical Rev. Biotechn. 6, 1987, 357-393; Timberlake and Marshall, Science 244, 1989, 1313-1317).

Usually, some transformants contain multiple copies of the transforming DNA. These multiple copies are scattered throughout the genome or they are organised in a tandem array at a single locus. Typical multiple copy transformants contain both scattered and tandem integration patterns of transformed DNA. Sometimes, rearrangements have occurred in the transformed DNA. Although transformation by integration of the transforming DNA into the genome is the rule, it should be noted that stable transformants might also be obtained by stable extra-chromosomal maintenance of the transforming DNA. Such a situation of stable extra-chromosomal maintenance can be obtained when sequences necessary for autonomous replication (ars) and/or other sequences necessary for stable extra-chromosomal replication are part of the transforming DNA. These sequences might be added to the transforming DNA by conventional genetic-engineering techniques prior to the transformation process, or alternatively, it can be conceived that ars sequences are selected from the genome by in vivo integration and xcision events during the transformation process (Pow ll and Kistl r, J. of Bacteriol. 172, 1990, 3163-3171). It can be envisaged also that the transforming DNA is designed to function as an artificial

chromosome in Penicillium chrysogenum, analogous to yeast artificial chromosomes (Burke et al., Science 236, 1987, 806-812) by the addition of centromere and telomere sequences, functional in P. chrysogenum, to the transforming DNA. Preferably, this invention relates to the stable transformation of P. chrysogenum, by stable integration of the transforming DNA into the genome of P. chrysogenum.

The transforming DNA, usually referred to as vector, typically consists of the following functional elements:

- an origin of replication functional in E. coli, which is necessary for plasmid propagation in E. coli;
- a selectable marker functional in E. coli, preferably not a β-lactamase gene, under control of appropriate E. coli expression signals, which is necessary for plasmid maintenance and transformant selection in E. coli;
- a selectable marker functional in P. chrysogenum, preferably the facA gene of P. chrysogenum under control of appropriate P. chrysogenum expression signals, which is necessary for the selection of transformants in P. chrysogenum. Expression signals are defined herein as signals necessary and sufficient for efficient initiation and termination of transcription and efficient initiation and termination of translation.

The selection marker is preferably expressed from its own, endogenous expression signals, although it is envisaged that appropiate expression of the marker might also be achieved by expression signals of other P. chrysogenum genes, e.g. the expression signals of the P. chrysogenum phosphoglycerate kinase (pgk) gene (Van Solingen et al., Nucl. Acid Res. 16, 1988, 11823) or orotidine-5'-phosphate decarboxylase (pyrG) gene, (EP-A-260762) or even by heterologous, non-P. chrysogenum expression signals obtained for instance from the A. nidulans glyceraldehyde-3-phosphate dehydrogenase (gpdA) gene (Punt et al., Gene 56, 1987, 117-124).

- Optionally, the vector contains also the phage lambda cos sequence which is necessary for efficient in vitro packaging of the recombinant DNA into phage particles.

- In another option, the vector contains also a sequence which acts to enhance the efficiency of transformation of P. chrysogenum, like the ans-1 sequence of A. nidulans (Ballance and Turner, Gene 36, 1985, 321-331; Cantoral et al., Bio/technology 5, 1987, 494-497) or the pyrG sequence of P. chrysogenum (EP-A-260762).

- In yet another option, the vector contains one or more, non-selectable DNA sequences of interest.

Introduction of non-selectable DNA into P. chrysogenum occurs most efficiently when the non-selectable DNA is physically linked to a selectable marker. However, this linkage is not a prerequisite for transformation of non-selectable DNA. It is also possible to introduce non-selectable DNA and a selectable marker into P. chrysogenum by using distinct DNA molecules. Depending on the selection system used and on the molar ratio of distinct DNAs used for transformation, co-transformation frequencies obtained with distinct DNA molecules, range from a few % up to approximately 90% (see for example Kolar et al., Gene 62, 1988, 127-134). The present invention relates also to the application of co-transformation strategies of Penicillium chrysogenum with non-selectable DNA. Co-transformation is defined in the present application as transformation of the selection marker together with non-selectable DNA which is physically linked or not to the selection marker, in the presence or absence of vector sequences. The non-selectable DNA is preferably derived from P. chrysogenum but it is envisaged that in the application of the invention the non-selectable DNA can be derived from a source other than P. chrysogenum. It should be noted that all sequences necessary for efficient manipulation, stable maintenance and replication of the vector in E. coli are not required for the selection of transformants of P. chrysogenum. Therefore, these sequences can be removed from the transforming DNA prior to transformation of P. chrysogenum, for instance by digestion with appropriate restriction enzym s and purification by gel-electrophoresis.

In a preferred embodiment of the invention th transforming DNA consists entirely of homologous, P. chrysogenum derived, sequences.

In another preferred embodiment of the invention the generation of stable transformants of P. chrysogenum is achieved by transformation of acetate non-utilizing industrial strains of P. chrysogenum. It is another preferred embodiment that acetate non-utilizing industrial strains of P. chrysogenum are obtained without mutagenesis, by positive selection for spontaneously resistance to fluoroacetate. The fluoroacetate resistant, acetate non-utilizing strains may be mutated at facA, facB or facC loci, analogous to the facA, facB and facC loci of A. nidulans and the acu-1, acu-11 and acu-12 loci of C. lagopus. In a still preferred embodiment of the invention the generation of stable transformants of P. chrysogenum is achieved by transformation of acetate non-utilizing industrial strains, mutated at the facA locus, with recombinant DNA containing the P. chrysogenum facA gene as a homologous selection marker.

In yet another preferred embodiment of the invention, transformants are assayed for complementation of the facA mutation by direct selection on medium containing acetate, although the possibility is recognized that other carbon sources like ethanol and the like which require acetyl CoA synthetase activity for catabolism might be used as well in the selection of FacA$^+$ transformants. A preferred embodiment of the invention is also the repeated application of the facA selection system to industrial strains of P. chrysogenum which have already been transformed using this selection system. The mutant facA genotype required for following transformation events, can be obtained by disruption or replacement of the wild type facA gene in the transformant by using the cloned facA gene of P. chrysogenum, but is preferably obtained by positive selection for spontaneous resistance to fluoroac tate.

The following non-limitative exampl s will illustrate the invention.

Exampl 1

Isolation of acetate non-utilizing mutants of P. chrysogenum

Positive selection for resistance to fluoroacetate has been used for the isolation of mutants of several strains of P. chrysogenum, one of them being P. chrysogenum strain P2 (ATCC 48271 (Lein, in: Overproduction of Microbial Metabolites, Vanek and Hostálek (eds) 1986, Butterworth Publishers, 105-139; Barredo et al., Curr. Genet. 16, 1989, 453-459)). These mutants are unable to utilize acetate as a carbon source. Approximately 10^6-10^7 spores were plated onto 25 ml of solidified selective medium of the following composition (per 1000 ml, pH 6.5): glucose, 5 g; $NaNO_3$, 2 g; KCl, 1 g; $KH_2PO_4.3H_2O$, 3 g; $MgSO_4.7H_2O$, 0.5 g; fluoroacetate (Aldrich) 10 g and agar (Oxoid No 3), 15 g and 1 ml of a trace-element solution which contained per 1000 ml: $ZnSO_4.7H_2O$, 22 g; H_3BO_3, 11 g; $MnCl_2.4H_2O$, 5 g; $FeSO_4.7H_2O$, 5 g; $CoCl_2.6H_2O$, 1.7 g; $CuSO_4.5H_2O$, 1.6 g; $Na_2MoO_4.2H_2O$, 1.5 g; EDTA, 5 g.

Fluoroacetate resistant (fac) colonies were purified on selection medium and subsequently tested for their inability to grow on acetate medium. Acetate medium consisted of the minimal medium described above with the modification that potassium acetate, in a concentration of 100 mM, replaced glucose and fluoroacetate. All incubations were at 25°C.

Stable acetate non-utilizing mutants (reversion frequency $\leq 10^{-7}$, tested on acetate medium) were obtained for Penicillium chrysogenum at a frequency of approximately 1.10^{-6}.

Example 2

Acetyl-CoA synthetase activity in acetate non-utilizing strains of P. chrysogenum

Fluoroacetate resistant, acetate non-utilizing strains of P. chrysogenum P2 were further characterized biochemically by

measurement of the acetyl-CoA synthetase activity. The strains were grown in shake flasks for 48 hours in a standard production medium, described in EP-A-357119. Then, the mycelium was harvested, lyophilized and ground in a mortar. Approximately, 0.4 g of ground mycelium was extracted for 45 minutes at 4°C with 10 ml of a buffer containing Tris-HCl, 100 mM pH 7.3; EDTA, 0.4 mM; DTT, 0.1 mM and PMSF, 0.1 mM. Cell-free extracts were obtained by centrifugation of the extract for 25 minutes at 12.000 g. Acetyl-CoA synthetase activity was determined immediately after preparation of the cell-free extracts by measurement of acetate dependent depletion of CoA with Ellman reagent [5,5' dithio-bis-(2-nitrobenzoic acid)] (DTNB), basically according to procedures described by Takao (Takao et al., Agric. Biol. Chem. 51, 1987, 145-152). To 750 µl of mixture A, containing Tris-HCl (200 mM, pH 8.0), KCl (100 mM) and $MgCl_2$ (20 mM) 150 µl of mixture B, containing ATP (40 mM), LiCoA (15 mM) and acetate (20 mM) was added. The assay was started by the addition of 600 µl of cell-free extract to this mixture. The assay was performed at 30°C. At different time-intervals aliquots (150 µl) were removed from the reaction mixture. The aliquots were added to 100 µl of TCA (10% w/v). The solution was then neutralized with 100 µl of 0.6 N NaOH and buffered with 1.2 ml phosphate (0.2 M, pH 7.4). Subsequently, 100 µl of a DTNB solution (4 mg/ml in 0.2 M phosphate buffer, pH 7.4) was added. The extinction of the colour reaction was measured at 413 nm with a spectrophotometer (LKB) after centrifugation of the sample for 5 minutes at 3000 rpm (Heraeus labofuge M). Typical results, presented in Table 1, show that a large proportion of the acetate non-utilizer mutants are deficient in acetyl-CoA synthetase activity or have greatly reduced levels of this enzymatic activity.

Tabl 1: Relative activity of Acetyl-CoA synthetase in P. chrysogenum P2 and some acetate non-utilizing derivatives of P2 expressed in arbitrary units per mg of protein per minute.

Strain		Acetyl-CoA synthetase activity (arbitrary units)
P2		100
P2-acetate non-utiliser	1	nd
	2	14
	3	18
	4	10
	5	nd

nd: not detectable.

Example 3

Isolation and characterization of the P. chrysogenum facA gene

Chemically synthesized facA oligonucleotide probes were tested on Southern blots containing restriction enzyme digests of chromosomal DNA of P. chrysogenum P2 (not shown). Oligonucleotides were labelled at their 5' end using γ-[^{32}P]-ATP and T4-polynucleotide kinase following standard procedures (Maniatis et al., in: Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratory, 1982 and 1989 (second edition)). The sequences of the oligonucleotides were derived from conserved regions in the nucleotide sequences of the A. nidulans facA gene and the homologous N. crassa acu-5 gene (Connerton et al., Molec. Microbiol. 4, 1990, 451-460).
Hybridization and washing of the blots was performed at 56°C using 6xSSC (0.9 M sodium chloride, 0.09 M sodium citrate) in the final wash.

Mixed probe facA7 (5' GATGGCCTC$^{G}_{A}$GGAATCATGGGAAGGTAGAT 3') generated a unique hybridization signal and was subsequently used for the screening of a genomic library of P. chrysogenum which has been made by methods well known in the art (Maniatis et al., ibid).

The facA gene of P. chrysogenum was isolated and characterized using standard procedures as described by Maniatis et al. (ibid).

DNA of some of the positively hybridizing phages has been purified. This DNA was further characterized by restriction enzyme analysis. The position of the facA gene on the cloned P. chrysogenum DNA in these phages has been determined by Southern blot analysis of restriction enzyme digests with facA specific oligonucleotide probes. In a control experiment, identical hybridizing fragments have been detected in chromosomal DNA of P. chrysogenum. By these means, the 6.5 kb PstI fragment present in phage facA7 (Figure 1) has been identified as a suitable fragment for subcloning of the facA gene in the vector pBluescript® II (Stratagene, La Jolla). The resulting plasmid has been named pPC2-3.

The facA gene was further characterized by nucleotide sequence analysis, see Sequence listing 1. Comparison of this nucleotide sequence with the nucleotide sequences of the facA gene of A. nidulans revealed a 80% homology. The amino acid sequence of acetyl-CoA synthetase of P. chrysogenum deduced from the nucleotide sequence (Sequence listing 1 and 2) is 89% homologous (including conservative amino acid changes) to the sequence of the A. nidulans acetyl-CoA synthetase (Connerton et al., ibid). Homologies have been determined by using MicroGenie™ version 7.0 sequence analysis software (Beckman).

Example 4

Transformation of Penicillium chrysogenum facA strains

P. chrysogenum facA strains were grown in 500 ml of a complete medium (YPD; 1% yeast extract, 2% peptone, 2% glucose) in a 2 l conical flask, by inoculating the medium with 2.10^6 spores per ml and subs quent incubation for 18 hours in a rotating incubator at 25°C and 300 rpr. After this incubation period, the mycelium was harvested by filtration of the medium

through miracloth filtration wrap (Calbiochem). The mycelium was washed with 50 ml of sterile wash buffer containing 0.63 M NaCl and 0.27 M $CaCl_2$ in destilled water and excess buffer was removed by blotting the filter containing the mycelium between towels. The mycelium was weighed in a sterile tube and transferred to a 500 ml conical flask, to which 20 ml of a buffer (0.53 M Nacl, 0.27 M $CaCl_2$) containing 100 mg Novozym 234 (NOVO Nordisk) was added per gram mycelium. Protoplasts were allowed to form by incubation at 25°C and gentle shaking (80 rpm) for 30-60 minutes, which process was followed microscopically. Free protoplasts were harvested by filtration of the suspension through glasswool, washing with an equal volume cold STC/0.63 M NaCl buffer (1.2 sorbitol, 10 mM Tris/pH 7.5, 50 mM $CaCl_2$) and subsequent centrifuging at 2500 rpm, 4°C in 50 ml conical tubes using a swing-out rotor. The protoplasts were resuspended twice in 50 ml of STC/0.63 M NaCl buffer and centrifuged. Subsequently, the protoplasts were resuspended in a small volume of 0.7 M KCl buffer and the concentration of the protoplasts was determined using a haemocytometer. Finally, the protoplasts were diluted at a concentration of 10^8/ml of STC/0.53 M NaCl and maintained on ice.

Aliquots of 100 µl of protoplasts suspension were added to sterile round bottom plastic tubes containing 10 µg linear or circular pPC2-3 DNA. After gentle mixing, the suspensions of protoplasts and DNA were incubated for 25 minutes at room temperature after which period a total volume of 1250 µl of a solution of polyethylene glycol (PEG) was added (60% PEG 4000 (BDH), 10 mM Tris/pH 7.5, 50 mM $CaCl_2$). The PEG solution was added as two aliquots of 200 µl, and one aliquot of 850 µl, with gentle but thorough mixing between each addition. This was followed by an incubation period of 20 minutes at room temperature. After incubation, the tubes were filled with 0.7 M KCl buffer and the protoplasts were spun down at 2500 rpm, 4°C. Subsequently, the protoplasts were plated on agar plates, containing 0.9 M KCl, 50 mM KAc, 0.001% glucose and minimal medium salts. The results of a typical experiment are given in

Table 2. The vector pBluescript has been used as a negative control.

Table 2: Number of FacA$^+$ transformants obtained with pPC2-3

DNA	µg	No. of transformants
pBluescript	10	0
pPC2-3	10	40

This result has been obtained by using various high-producing strains of P. chrysogenum, among them P. chrysogenum strain P2. It will be well known to those skilled in the art that the procedures for transformation require minor adjustments depending on the particular P. chrysogenum strain used.

Transformants usually sporulated within 7 days of incubation at 25°C on the medium described above.

In pPC2-3 transformed strains acetyl-CoA synthetase activity (determined according to Example 2) was restored to or above wild type levels (Table 3).

Table 3: Relative activity of Acetyl-CoA synthetase in two FacA$^+$ transformants and P2 expressed in arbitrary units per mg of protein per minute.

Strain	Acetyl-CoA synthetase activity (arbitrary units)
P2	100
FacA$^+$ transformant 1	200
2	1000

Example

DNA analysis of obtained transformants

To verify the presence of intact vector sequences in the chromosomal DNA of the obtained FacA$^+$ colonies and to identify transformants having only one copy of the v ctor integrated,

DNA of 14 individual colonies was purified and analyzed by Southern hybridization. DNA of the colonies was isolated as follows. Complete medium as described in Example 4 (50 ml thereof in 250 ml conical flasks) was inoculated with 10^8 spores of each colony, obtained after two cycles of single spore inoculations on minimal medium plates containing 100 mM KAc as sole carbon source. The medium was incubated at 300 rpm on a rotary shaker at 25°C for 48 hours after which the mycelium was harvested using miracloth filtration wrap and washed with 25 ml of a 0.9% NaCl solution. Then the mycelium was weighed and frozen immediately in liquid nitrogen. Subsequently, portions of the mycelium were ground using a mortar and a pestle, while liquid nitrogen was added repeatedly, until a fine powder was obtained. The powder was added to a DNAse-free tube to which 10 ml of the extraction buffer was added per gram mycelium. The extraction buffer was prepared as follows: 40 ml of ice-cold 5xRNB buffer (1.0 M Tris-HCl, pH 8.5, 1.25 M NaCl, 0.25 M EDTA, autoclaved) was added to 80 ml of ice-cold p-aminosalicylic acid (123 g/l; Sigma) to which 80 ml ice-cold TNS (tri-isopropylnaphtalenesulfonic acid, sodium salt; 20 g/l; Eastman Kodak) was added. After mixing, a precipate was allowed to form on ice, from which the upper fluid was used for extraction of the mycelium.

After addition of the frozen mycelium powder to the extraction buffer the mycelium was allowed to thaw by vortexing and 0.5 volumes of phenol solution 1 was added immediately. Phenol solution 1 was prepared by dilution of phenol crystals in demineralized water and subsequent adjustment of the pH to 8.0 with NaOH solution. After addition of phenol solution 1, the mycelium suspension was mixed thoroughly and incubated on ice until the last mycelium sample was ground. Then, 0.5 volumes of chloroform was added to each tube and the tubes were mixed once again.

Next, the tubes were centrifuged at 12000 rpm, 4°C for 10 minutes, using a swing-out rotor. The upper phases, containing the DNA, were transferred to new tubes to which 10 ml of phenol solution 2 was added. Phenol solution 2 was prepared by

diluting 100 g phenol in 100 ml 25:1 (v/v) of chloroformisoamylalcohol. Then 1.6 mg of 8-hydroxyquinoline was added and the solution was saturated with STE (0.3 M NaCl, 10 mM Tris/pH 7.5, 0.1 mM EDTA).

After vortexing, the tubes containing the DNA were centrifuged once again and the upper phase transferred to another tube. Subsequently 3 volumes of 96% ethanol (stored at -20°C) were added and the DNA was allowed to precipitate for 30 minutes at -70°C. The tubes were centrifuged at 20000 rpm for 15 minutes, 4°C and the DNA pellets were washed with 70% ethanol (stored at -20°C). The pellets were dried in a vacuum exsiccator for 3 minutes, resuspended in 0.5-1.0 ml of STE, depending on the size of the pellet, and transferred to eppendorf tubes. To each tube 10 µl of a 20 mg/ml RNAse A solution was added and the tubes were incubated for 15 minutes at 37°C. The DNA solutions were extracted again with phenol solution 2 for two or three times and the DNA was precipitated as described above. Finally, the washed pellets were dissolved in TE buffer (10 mM Tris/ pH 7.5; 0.1 mM EDTA).

The procedure followed to analyse the chromosomal DNA by Southern hybridization was essentially carried out as described in Maniatis et al. (1982). DNA was digested with PstI, followed by separation of fragments on a 0.6% agarose gel and then transferred to nitrocellulose sheets. These blots were hybridized with either ^{32}P labelled DNA of pPC1-1 or with the ^{32}P labelled PstI fragment containing the P. chrysogenum facA gene (Figure 2,A). Hybridization and washing of the blots was carried out at 65°C using 0.2 x SSC in the final wash. After exposure of the blots to X-ray sensitive films the patterns of hybridization obtained were analyzed (Figure 2,B). From this analysis it could be concluded that all but 2 transformants contain vector fragments. The pattern of these two transformants (Nos. 7 and 11) is indistinguishable of the wildtype pattern, which probably indicates that they aros after replacement or conversion of the mutant allele. Six transformants (Nos. 1, 2, 3, 6, 10 and 12) contain a single copy of the vector at the resident facA locus wher as four

(Nos. 4, 8, 13 and 14) contain a single vector copy at an unknown genomic site. Two transformants (Nos. 5 and 9) contain multiple copies of the vector. This experiment demonstrated that the facA transformant selection system is a versatile selection system, suitable for different applications like for example the generation of single-copy transformants, the generation of multi-copy transformants, integration at the resident facA locus, or integration at unknown genomic sites.

Example 6

Penicillin production of facA mutants of P. chrysogenum

The effect of the fluoroacetate selection procedure on penicillin production has been determined for 3 stable facA mutants, obtained from approximately 3.10^6 spores as has been described in Example 1. The production of penicillin was determined in shake flask experiments, in two independent experiments, using procedures which have been described before in EP-A-357119. The results are summarized in Table 4.

Table 4: Penicillin production of facA mutants of Penicillium chrysogenum. The amount of penicillin is expressed in arbitrary units. The number of arbitrary units produced by P2 is arbitrarily set at 100.

Strain	Penicillin production (arbitrary units)
P2	100
P2 facA1	103
P2 facA5	91
P2 facA7	109

This experiment shows that facA mutants with unaltered penicillin production characteristics are readily obtained. FacA$^+$ transformants with unchanged l vels of penicillin production compared to the parent strain P2 were also readily obt-

ained by transformation of the facA mutants with pPC2-3 (not shown).

Example 7

Repeated use of the facA transformation system

A P. chrysogenum facA strain was transformed with pPC2-3. Transformants containing a single copy of the transforming facA gene were identified, as has been described in Example 5. Such a transformant was subjected to a second round of (1) selection for acetate non-utilising facA mutants and (2) a second transformation with pPC2-3 as has been described in Example 4. Acetate non-utilizing mutants were obtained by positive selection on minimal medium containing fluoroacetate as has been described in Example 1. The frequency of occurrence of fluoroacetate resistant, acetate non-utilizing colonies was comparable to the frequency observed with the parent strain P2.

Second generation facA mutants, identified as has been described in Example 2, behaved in a similar way in transformation experiments, in stability tests and in penicillin production tests as has been described for first generation facA mutants (see Examples 4, 5 and 6). The experiments described here demonstrate that efficient repeated application of the facA transformation system is possible.

Example 8

Homologous transformation

The feasibility of complete homologous transformation by using the 6.5 kb Pst I restriction fragment of pPC2-3 is demonstrated.

Plasmid pPC2-3 was propagated by using E. coli strain JM109 (Yanish-Perron et al., Gene 33, 1985, 103-109) and

purified according to methods well known in the art (Maniatis et al., ibid.). The purified plasmid pPC2-3 was then digested with restriction enzyme Pst I (New England Biolabs) to liberate the P. chrysogenum derived sequences from pBluescript vector sequences. The fragment containing the P. chrysogenum derived sequences, 6.5 kb in length, was purified from pBluescript vector sequences by agarose gel electrophoresis followed by electroelution from the agarose gel (Bio-trap™, Schleicher and Schuell).

The purified 6.5 kb Pst I restriction fragment was then used for transformation of facA strains of P. chrysogenum according to procedures described in Example 4. Transformation frequencies were similar to those obtained by using the entire plasmid pPC2-3 (Table 5).

Table 5: Number of FacA$^+$ transformants

DNA	µg	No. of transformants
pBluescript	10	-
pPC2-3	10	40
6.5 kb Pst I fragment	10	50

The absence of pBluescript vector sequences were subsequently demonstrated by using a sensitive colony-hybridization procedure (Kinsey, Fungal Genetics, Newslett. 36, 1989, 45-47) and by using randomly labeled pBluescript as a probe (Maniatis et al., ibid.).

Example 9

FacA mediated co-transformation

The possiblity to introduce non-selectable DNA into P. chrysogenum by using the facA selection system is demonstrated by the control experiment described in this example.

A P. chrysogenum facA mutant was transformed with the 6.5 kb PstI restriction fragment as described in example 8 together

with a 3 kb P. chrysogenum derived SalI restriction fragment which contains an oligomycine resistant oliC gene. Such an oliC gene can be obtained from P. chrysogenum by methods described in detail (Bull et al., Curr. Genet. 1988, 13, 377-382).

Transformants were selected first for growth on acetate containing medium as described in Example 4. Then, following purification of transformants on acetate medium, transformants were tested for resistence to oligomycine by growth on solid medium containing 3 µg/ml oligomycine (Sigma). Oligomycine resistant transformants were readily obtained using a 1:1 molar ratio of facA/oliC DNAs.

From these results it is concluded that co-transformation readily occurs by using the facA selection system. Physical linkage of the selection marker to the non-selectable DNA is not required for co-transformation.

Example 10

Selection on ethanol containing media

The possibility to select FacA$^+$ transformants on medium containing ethanol rather than acetate as a carbon source is demonstrated. FacA$^+$ transformants were obtained by procedures described in Example 4. The transformation mixture was plated on selection medium containing 0.1%, 0.3% or 1% ethanol instead of 50 mM potassium acetate. Otherwise, the selection medium used in this example is identical to the medium described in Example 4.

After approximately 2-3 weeks of incubation at 25°C transformants could clearly be identified. By using ethanol containing selection medium the frequency of transformation was reduced to approximately 0.5 - 1 transformants/µg of DNA.

This example demonstrates the feasibility to use other carbon sources than acetate, which require acetyl-CoA synthetase activity for catabolism.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Gist-brocades N.V.
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(ii) TITLE OF INVENTION: Transformant Selection System for β-Lactam Producing Strains

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM: WordPerfect 5.0

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: -
(B) FILING DATE: 15 October 1991

(vi) ATTORNEY/AGENT INFORMATION:
(A) NAME: Visser-Luirink, Gesina, Dr.
(C) REFERENCE NUMBER: PCT-2564

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(A) TELEPHONE: 015-793940
(B) TELEFAX: 015-793957

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4652 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Penicillium chrysogenum

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPC2-3

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1781..1819

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1820..1904

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1905..3149

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 3150..3207

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 3208..3468

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 3469..3519

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 3520..3648

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 3649..3709

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 3710..3981

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 3982..4057

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 4058..4117

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join (1781..1819, 1905..3149, 3208..3468, 3520..3648, 3710..3981, 4058..4117)
(D) OTHER INFORMATION: /codon_start= 1781
/gene= "facA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGACAAA AGGAACGAGA ACTAAAGGAT GCATCCGCCT GTTATCAAAC ATATGATGTG 60

GTCATGACGG CCCTTGTTCC TAAACTTCGT CCTGCAAGAT TACTTGATCC GACCCTTTTT 120

CGACTCTTCC ACTACTGTCA CACAACTTGG AGAGATTCGG CTGTCGCTGT CCGCCAAGAG 180

TTAATTGAGC TTTCAGCTCG CTGGGCGGAA CTGGGGCTAG AGGGCTCATG TCCATATTCT 240

CCCACAGAAG CGGAATTGAA ACAGCACGCT CGAGACTATG AAGATTTTGA GGCTGTTCAA 300

GCACTCAAGT CATGGCTAAG GGACAACCTT GATACAAATT CTGACGGGTG GATCTCGAGT 360

GATGCGTGGG ACGCTGCGAG GGTAGCTCAT CGCGCAGCGT ATGATGAATG GATACAGACT 420

GCCAAAGAAG CCGAGTCCCG TGGCGAAGAC ATGACAGTAG CGAAGGCGGA CAAGATGTGG 480

CCATTTGATG CTAGATAGGA ACTAGGGAGA GGTAATCAAA ATAGCCAGCT GGTCTCTGTC 540

TCTAGGGTCT AGTCTAAACC CAGGCCCTGC CCTTACGTTA GCTTTTCGAC AGGATATGAC 600

GGCCTGATGT TTGTTTCTGC CTGGCTAGGC AATTTTTAAG TAGATCTCGG CTTTGGGTGC 660

GTAGCTTCTG GGAACTTGGT TGGCTCCTCT ACGTCTGAAC AGCTCCTTGG CTGTTGTAGA 720

TGTCGTAGCA GTATATTCAA CCGTACAATA TTTACAATAG TAGTCCAAAG GGTCTCGAGC 780

TTAATGATAC TCCGGCCCCG TCTAAAAGTC ATGTGGTCCA CGCGTTAGCG CGCCGTTAGT 840

CTAAGTATAC GACATATGTA CGACAGACAA GGCCACATGG TGAATTTAGC GCAGGTGTGA 900

CTTTAAGTGG ACCGGCACTG GACGGGTTTG GCGCGAAATA CAGTGCTTTT CTCAACATGT 960

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TTTTGTTGGG ACATTGAGCA AAGAATGGAG TAAACGCGGT CTTTTCTCGG GCCTCTTTCC 1080

TCTGCGTTAA AAATTGGCGG GGCTCGAAGA TACGCTTGTA TTGAAACTGG CTTCTCCCCT 1140

GATCCGCGAC CCCGGACCTT GGCTGAAGTA CCTAGGTTGT GTTGGACTCG GACAAAGGGT 1200

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AACGGAAAGA ACCCCCGCAT GGCCGAACCC AAACTCGTAT GGGACAAGGC AATTTACTGA 1380

AATTTACTGA AATTTACTGA ATTGGACCGT ATTCGGAATG TATCTTATTC CTGATTCGGA 1440

GATGAGAGTG GATCGTCCGA ATGTCCAATG CACAATGTAC TTTCTCTAGG CCGTCTGCGG 1500

CTAGCGAGAC AGCCGGAGTT GGGTAGTTTG AAGTGGTATT GTAACTTATT GTAATTTATT 1560

GTAAGGGGCA CGGACCACTG ATGAAAAGGG AAGTGGCACA TCCTCCCGGG ACAGCTGGAC 1620

TACTAATATT GTCGCGAGTC CCCCCTCCTT GAGTTTCTTT TTCTCTTTCT CTTCTCTAAT 1680

ATCTTCTCTA ATTGCTATAC ATACCCTGTT TGATCATTAC TCTTAGTATA TTATATAGTT 1740

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Met Ser Asp Gly Pro
1 5

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                                                            Val
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His Glu Ala His Glu Val Asp Thr Phe His Val Pro Lys Ala Phe His
 15                 20                  25                  30

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Asp Lys His Pro Ser Gly Thr His Ile Lys Asp Ile Glu Glu Tyr Lys
                35                  40                  45

AAG CTT TAC GAA GAA TCA ATC AAG AGC CCC GAC ACC TTC TGG GCA CGC      2051
Lys Leu Tyr Glu Glu Ser Ile Lys Ser Pro Asp Thr Phe Trp Ala Arg
            50                  55                  60

ATG GCC CGC GAG CTC CTC ACA TTT GAC AAG GAC TTT GAA ACC ACA CAT      2099
Met Ala Arg Glu Leu Leu Thr Phe Asp Lys Asp Phe Glu Thr Thr His
        65                  70                  75

CAC GGC TCG TTT GAG AAC GGC GAC AAT GCC TGG TTC GTC GAG GGT CGG      2147
His Gly Ser Phe Glu Asn Gly Asp Asn Ala Trp Phe Val Glu Gly Arg
    80                  85                  90

TTG AAC GCA TCG TTC AAC TGT GTC GAT CGC CAT GCC CTC AAG AAC CCA      2195
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 95                 100                 105                 110

GAT AAG GTC GCC ATT ATT TAT GAG GCC GAC GAG CCC AAC GAG GGC CGT      2243
Asp Lys Val Ala Ile Ile Tyr Glu Ala Asp Glu Pro Asn Glu Gly Arg
                115                 120                 125

AAG ATC ACC TAC GGA GAG CTG ATG CGC GAG GTG TCC CGG GTT GCC TGG      2291
Lys Ile Thr Tyr Gly Glu Leu Met Arg Glu Val Ser Arg Val Ala Trp
            130                 135                 140

ACT CTG AAG GAG CGT GGC GTC AAG AAG GGC GAC ACG GTC GGT ATC TAC      2339
Thr Leu Lys Glu Arg Gly Val Lys Lys Gly Asp Thr Val Gly Ile Tyr
        145                 150                 155

CTG CCC ATG ATT CCC GAG GCC GTA ATC GCT TTC CTG GCT TGC TCG CGT      2387
Leu Pro Met Ile Pro Glu Ala Val Ile Ala Phe Leu Ala Cys Ser Arg
    160                 165                 170

ATT GGT GCC GTG CAC TCC GTT GTC TTC GCT GGT TTC TCT TCC GAC TCC      2435
Ile Gly Ala Val His Ser Val Val Phe Ala Gly Phe Ser Ser Asp Ser
175                 180                 185                 190

CTC CGG GAC CGT GTC CTG GAC GCC TCC TCC AAG GTC ATC ATT ACC TCC      2483
Leu Arg Asp Arg Val Leu Asp Ala Ser Ser Lys Val Ile Ile Thr Ser
                195                 200                 205
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GAC GAG GGC AAG CGC GGT GGC AAG ATC ATT GGC ACT AAG AAG ATT GTG 2531
Asp Glu Gly Lys Arg Gly Gly Lys Ile Ile Gly Thr Lys Lys Ile Val
210 215 220

GAC GAG GCC ATG AAG CAG TGC CCC GAT GTC GAC ACC GTG CTG GTG TAC 2579
Asp Glu Ala Met Lys Gln Cys Pro Asp Val Asp Thr Val Leu Val Tyr
225 230 235

AAG CGC ACC GGT GCC GAG GTG CCC TGG ACC GCT GGC CGT GAC ATT TGG 2627
Lys Arg Thr Gly Ala Glu Val Pro Trp Thr Ala Gly Arg Asp Ile Trp
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TGG CAC GAG GAG GTC GAG AAG TAC CCC AAC TAC CTC GCC CCT GAG TCG 2675
Trp His Glu Glu Val Glu Lys Tyr Pro Asn Tyr Leu Ala Pro Glu Ser
255 260 265 270

GTC AGC TCC GAG GAT CCT CTC TTC CTG TTG TAC ACC TCC GGT TCC ACC 2723
Val Ser Ser Glu Asp Pro Leu Phe Leu Leu Tyr Thr Ser Gly Ser Thr
275 280 285

GGT AAG CCC AAG GGT GTT ATG CAC ACC ACT GCC GGT TAC CTG CTC GGT 2771
Gly Lys Pro Lys Gly Val Met His Thr Thr Ala Gly Tyr Leu Leu Gly
290 295 300

GCG GCC ATG ACT GGA AAG TAC GTG TTT GAT ATC CAC GAC GAT GAT CGC 2819
Ala Ala Met Thr Gly Lys Tyr Val Phe Asp Ile His Asp Asp Asp Arg
305 310 315

TAC TTC TGC GGT GGT GAT GTC GGT TGG ATT ACA GGT CAC ACC TAT GTC 2867
Tyr Phe Cys Gly Gly Asp Val Gly Trp Ile Thr Gly His Thr Tyr Val
320 325 330

GTG TAC GCC CCT CTA TTG CTT GGC TGC GCC ACC GTC GTG TTC GAG AGT 2915
Val Tyr Ala Pro Leu Leu Leu Gly Cys Ala Thr Val Val Phe Glu Ser
335 340 345 350

ACC CCC GCC TAC CCT AAC TTC TCG CGC TAC TGG GAT GTC ATT GAC AAG 2963
Thr Pro Ala Tyr Pro Asn Phe Ser Arg Tyr Trp Asp Val Ile Asp Lys
355 360 365

CAC GAC GTC ACA CAA TTC TAC GTT GCA CCC ACC GCT CTG CGT CTG CTG 3011
His Asp Val Thr Gln Phe Tyr Val Ala Pro Thr Ala Leu Arg Leu Leu
370 375 380

AAG CGC GCT GGA GAT GAG CAC ATT CAC CAC AAG ATG CAC AGT CTG CGT 3059
Lys Arg Ala Gly Asp Glu His Ile His His Lys Met His Ser Leu Arg
385 390 395

ATT CTT GGC TCC GTC GGA GAG CCC ATT GCC GCG GAA GTC TGG AAG TGG 3107
Ile Leu Gly Ser Val Gly Glu Pro Ile Ala Ala Glu Val Trp Lys Trp
400 405 410

TAC TTC GAG TGT GTT GGC AAG GAG GAA GCT CAC ATC TGC GAC 3149
Tyr Phe Glu Cys Val Gly Lys Glu Glu Ala His Ile Cys Asp
415 420 425

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GTTCGTTCCC CCTTACCCTT GGACCTTTTG GAATAACTTC TAATTTTTGG ATCTGTAG          3207

ACA TAC TGG CAA ACC GAG ACC GGC TCA CAT GTC ATC ACC CCT CTC GGC          3255
Thr Tyr Trp Gln Thr Glu Thr Gly Ser His Val Ile Thr Pro Leu Gly
    430                 435                 440

GGT ATC ACC CCC ACC AAG CCC GGC AGT GCC TCC CTA CCC TTC TTC GGT          3303
Gly Ile Thr Pro Thr Lys Pro Gly Ser Ala Ser Leu Pro Phe Phe Gly
445                 450                 455                 460

ATC GAG CCT GCC ATT ATC GAC CCC GTC TCC GGA GAG GAG ATT GTC GGC          3351
Ile Glu Pro Ala Ile Ile Asp Pro Val Ser Gly Glu Glu Ile Val Gly
                465                 470                 475

AAT GAT GTC GAG GGT GTT TTG GCC TTC AAG CAG CCG TGG CCC AGC ATG          3399
Asn Asp Val Glu Gly Val Leu Ala Phe Lys Gln Pro Trp Pro Ser Met
            480                 485                 490

GCC CGC ACC GTG TGG GGT GCC CAC AAG CGT TAC ATG GAC ACT TAC TTG          3447
Ala Arg Thr Val Trp Gly Ala His Lys Arg Tyr Met Asp Thr Tyr Leu
        495                 500                 505

AAC GTG TAC AAG GGT TAC TAC GTAAGACGCT TCGCAGCCTG CCTTGCAGGG             3498
Asn Val Tyr Lys Gly Tyr Tyr
    510                 515

TTGATACTAA CTCATATATA G TTC ACC GGA GAT GGT GCT GGC CGT GAC CAC          3549
                      Phe Thr Gly Asp Gly Ala Gly Arg Asp His
                                  520                 525

GAC GGC TAT TAC TGG ATC CGC GGT CGT GTT GAC GAT GTC GTC AAC GTT          3597
Asp Gly Tyr Tyr Trp Ile Arg Gly Arg Val Asp Asp Val Val Asn Val
                530                 535                 540

TCT GGA CAC CGT CTG TCC ACC GCT GAG ATC GAG GCC GCT CTT CTC GAG          3645
Ser Gly His Arg Leu Ser Thr Ala Glu Ile Glu Ala Ala Leu Leu Glu
            545                 550                 555

CAC  C GTAAGTCCAA CCACAGTATC TGCCAAAAAT TGCAACTGAG CCCAAACTAA           3699
His

CTATGAACAG  CT TCC GTT GCC GAG GCT GCT GTC GTT GGT ATT GCC GAC           3747
          Pro Ser Val Ala Glu Ala Ala Val Val Gly Ile Ala Asp
              560                 565                 570

GAG CTG ACC GGT CAG GCT GTC AAT GCC TTT GTC TCT CTC AAG GAG GGC          3795
Glu Leu Thr Gly Gln Ala Val Asn Ala Phe Val Ser Leu Lys Glu Gly
            575                 580                 585

AAG CCC ACA GAA CAG ATC AGC AAG GAC CTT GCA ATG CAA GTT CGC AAG          3843
Lys Pro Thr Glu Gln Ile Ser Lys Asp Leu Ala Met Gln Val Arg Lys
        590                 595                 600
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TCC ATT GGT CCC TTC GCC GCC CCC AAG GCT GTC TTC GTC GTG GAT GAC 3891
Ser Ile Gly Pro Phe Ala Ala Pro Lys Ala Val Phe Val Val Asp Asp
605 610 615

CTC CCC AAG ACC CGC AGT GGC AAG ATC ATG CGC CGA ATC CTC CGG AAG 3939
Leu Pro Lys Thr Arg Ser Gly Lys Ile Met Arg Arg Ile Leu Arg Lys
620 625 630 635

ATT CTC AGT GGC GAG GAG GAC AGC CTC GGT GAT ACA TCA ACG [illegible]
Ile Leu Ser Gly Glu Glu Asp Ser Leu Gly Asp Thr Ser Thr
640 645

GTAAGCATCA TCTCTCAGCA AGATAGTACC CGCAATCGTA TCGTCCGAAC AATAGCTAAC 4041

GAAATATTCT TCACAG CTC TCC GAC CCC AGT GTC GTG GAC AAG ATC ATA 4090
Leu Ser Asp Pro Ser Val Val Asp Lys Ile Ile
650 655 660

GAA ACC GTC CAC AGT GCT CGC CAG AAG TAAAGTGAAA GTCTATGAAT 4137
Glu Thr Val His Ser Ala Arg Gln Lys
665 670

ATGATGATAA TGACGTCCGA GAGCAAAATT TCTGGTGAAT TTTGGAAGTA GTATGATGGT 4197

CCTCTGCCGA TCATACGCCC TCGACCTCGG TCCACTTGGT TCATGCTGGA ATCGGACTTG 4257

ACCATGCGGG TGGTTTTCTT TTCTTTTCTT TTTTTGGCCG GTTTTCAGAA TCACTGCTTG 4317

TACTTGAGAT TCCCTTGGCT CGCTCAGAAG CGATTTGAAT AGTATTATTT TTTGCCTTCT 4377

TGTATACTTC GGCTCTCTCC TTTGACTCAT CAATATGAAT CGTACCTAGG TATAAGAGCA 4437

TCTTTACGGG TGGAGCCATT GACGGAACTC CATGACGCCG TTGAATGCGC CTTGAGCTAC 4497

TTATAGGGGG CCGGGGGATG TGGTAGAAGG CGATGGATCA TGACTTGAAA CCATACAGAT 4557

GCTGGTGCAG GACTGCACTG GGTTCCCGCG CGTATGCTTC TAATATAAAC GTTCTGTGAC 4617

GCATCTTTTC AATTCTGCGG AAGGGTCAAG AATTC 4652

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 669 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Asp Gly Pro Ile Gln Pro Pro Lys Pro Ala Val Val His Glu
1 5 10 15

Ala His Glu Val Asp Thr Phe His Val Pro Lys Ala Phe His Asp Lys
20 25 30

His Pro Ser Gly Thr His Ile Lys Asp Ile Glu Glu Tyr Lys Lys Leu
35 40 45

Tyr Glu Glu Ser Ile Lys Ser Pro Asp Thr Phe Trp Ala Arg Met Ala
50 55 60

Arg Glu Leu Leu Thr Phe Asp Lys Asp Phe Glu Thr Thr His His Gly
65 70 75 80

Ser Phe Glu Asn Gly Asp Asn Ala Trp Phe Val Glu Gly Arg Leu Asn
85 90 95

Ala Ser Phe Asn Cys Val Asp Arg His Ala Leu Lys Asn Pro Asp Lys
100 105 110

Val Ala Ile Ile Tyr Glu Ala Asp Glu Pro Asn Glu Gly Arg Lys Ile
115 120 125

Thr Tyr Gly Glu Leu Met Arg Glu Val Ser Arg Val Ala Trp Thr Leu
130 135 140

Lys Glu Arg Gly Val Lys Lys Gly Asp Thr Val Gly Ile Tyr Leu Pro
145 150 155 160

Met Ile Pro Glu Ala Val Ile Ala Phe Leu Ala Cys Ser Arg Ile Gly
165 170 175

Ala Val His Ser Val Val Phe Ala Gly Phe Ser Ser Asp Ser Leu Arg
180 185 190

Asp Arg Val Leu Asp Ala Ser Ser Lys Val Ile Ile Thr Ser Asp Glu
195 200 205

Gly Lys Arg Gly Gly Lys Ile Ile Gly Thr Lys Lys Ile Val Asp Glu
210 215 220

Ala Met Lys Gln Cys Pro Asp Val Asp Thr Val Leu Val Tyr Lys Arg
225 230 235 240

Thr Gly Ala Glu Val Pro Trp Thr Ala Gly Arg Asp Ile Trp Trp His
245 250 255

Glu Glu Val Glu Lys Tyr Pro Asn Tyr Leu Ala Pro Glu Ser Val Ser
260 265 270

Ser Glu Asp Pro Leu Phe Leu Leu Tyr Thr Ser Gly Ser Thr Gly Lys
275 280 285

Pro Lys Gly Val Met His Thr Thr Ala Gly Tyr Leu Leu Gly Ala Ala
290 295 300

Met Thr Gly Lys Tyr Val Phe Asp Ile His Asp Asp Asp Arg Tyr Phe
305 310 315 320

Cys Gly Gly Asp Val Gly Trp Ile Thr Gly His Thr Tyr Val Val Tyr
325 330 335

Ala Pro Leu Leu Leu Gly Cys Ala Thr Val Val Phe Glu Ser Thr Pro
340 345 350

Ala Tyr Pro Asn Phe Ser Arg Tyr Trp Asp Val Ile Asp Lys His Asp
355 360 365

Val Thr Gln Phe Tyr Val Ala Pro Thr Ala Leu Arg Leu Leu Lys Arg
370 375 380

Ala Gly Asp Glu His Ile His His Lys Met His Ser Leu Arg Ile Leu
385 390 395 400

Gly Ser Val Gly Glu Pro Ile Ala Ala Glu Val Trp Lys Trp Tyr Phe
405 410 415

Glu Cys Val Gly Lys Glu Glu Ala His Ile Cys Asp Thr Tyr Trp Gln
420 425 430

Thr Glu Thr Gly Ser His Val Ile Thr Pro Leu Gly Gly Ile Thr Pro
435 440 445

Thr Lys Pro Gly Ser Ala Ser Leu Pro Phe Phe Gly Ile Glu Pro Ala
450 455 460

Ile Ile Asp Pro Val Ser Gly Glu Glu Ile Val Gly Asn Asp Val Glu
465 470 475 480

Gly Val Leu Ala Phe Lys Gln Pro Trp Pro Ser Met Ala Arg Thr Val
485 490 495

Trp Gly Ala His Lys Arg Tyr Met Asp Thr Tyr Leu Asn Val Tyr Lys
500 505 510

Gly Tyr Tyr Phe Thr Gly Asp Gly Ala Gly Arg Asp His Asp Gly Tyr
515 520 525

Tyr Trp Ile Arg Gly Arg Val Asp Asp Val Val Asn Val Ser Gly His
530 535 540

Arg Leu Ser Thr Ala Glu Ile Glu Ala Ala Leu Leu Glu His Pro Ser
545 550 555 560

Val Ala Glu Ala Ala Val Val Gly Ile Ala Asp Glu Leu Thr Gly Gln
565 570 575

Ala Val Asn Ala Phe Val Ser Leu Lys Glu Gly Lys Pro Thr Glu Gln
580 585 590

Ile Ser Lys Asp Leu Ala Met Gln Val Arg Lys Ser Ile Gly Pro Phe
595 600 605

Ala Ala Pro Lys Ala Val Phe Val Val Asp Asp Leu Pro Lys Thr Arg
610 615 620

Ser Gly Lys Ile Met Arg Arg Ile Leu Arg Lys Ile Leu Ser Gly Glu
625 630 635 640

Glu Asp Ser Leu Gly Asp Thr Ser Thr Leu Arg Pro Gln Cys Arg Gly
645 650 655

Gln Asp His Arg Asn Arg Pro Gln Cys Ser Pro Glu Val
660 665

Claims

1. A method to select transformants of a microorganism which has been transformed with DNA which method comprises:

isolating a mutant of the microorganism in which acetyl-CoA synthetase is inoperable or absent;

cotransforming said mutant with said DNA and an expression system effective in producing acetyl-CoA synthetase of Penicillium chrysogenum; and

selecting transformants of said microorganism for ability to grow on a medium which contains a carbon source which requires acetyl-CoA synthetase activity for catabolism.

2. A method for obtaining or enhancing the production of a β-lactam compound by using transformants of a microorganism obtainable by the method of claim 1, which method comprises cotransforming said mutant with DNA encoding genetic information necessary for obtaining or enhancing the production of a β-lactam compound.

3. The method of claim 1 or 2 wherein said microorganism is Penicillium chrysogenum which has been transformed with homologous DNA.

4. The method of any one of the preceding claims wherein said carbon source is acetate.

5. The method of any one of the preceding claims wherein said cotransformation is conducted by supplying said DNA and said expression system on the same DNA molecule.

6. The method of any one of the preceding claims wherein said microorganism is a strain of fungus, preferably a β-lactam producing strain, more preferably Penicillium chrysogenum, Aspergillus nidulans or Acremonium chrysogenum.

7. The method of claim 6, wherein said β-lactam producing strain is a penicillin producing strain.

8. The method of any one of the preceding claims wherein said mutant is a spontaneous mutant identified by fluoroacetate resistance.

9. The method of any one of the preceding claims which further includes isolation of mutants lacking acetyl-CoA synthetase activity from said transformed mutant.

10. A facA gene which can be isolated from Penicillium chrysogenum.

11. A facA gene according to claim 10 having the nucleotide sequence depicted in Sequence listing 1.

12. The expression signals of the facA gene as defined in claim 10 or 11.

13. A gene according to claim 10 or 11 wherein one or more of said expression signals have been replaced by other expression signals, obtained from the same or another organism.

14. A vector comprising the facA gene as defined in claim 10, 11 or 13.

15. A transformed host comprising a facA gene as defined in claim 10, 11 or 13.

16. Use of a transformed host according to claim 15 to produce a β-lactam compound.

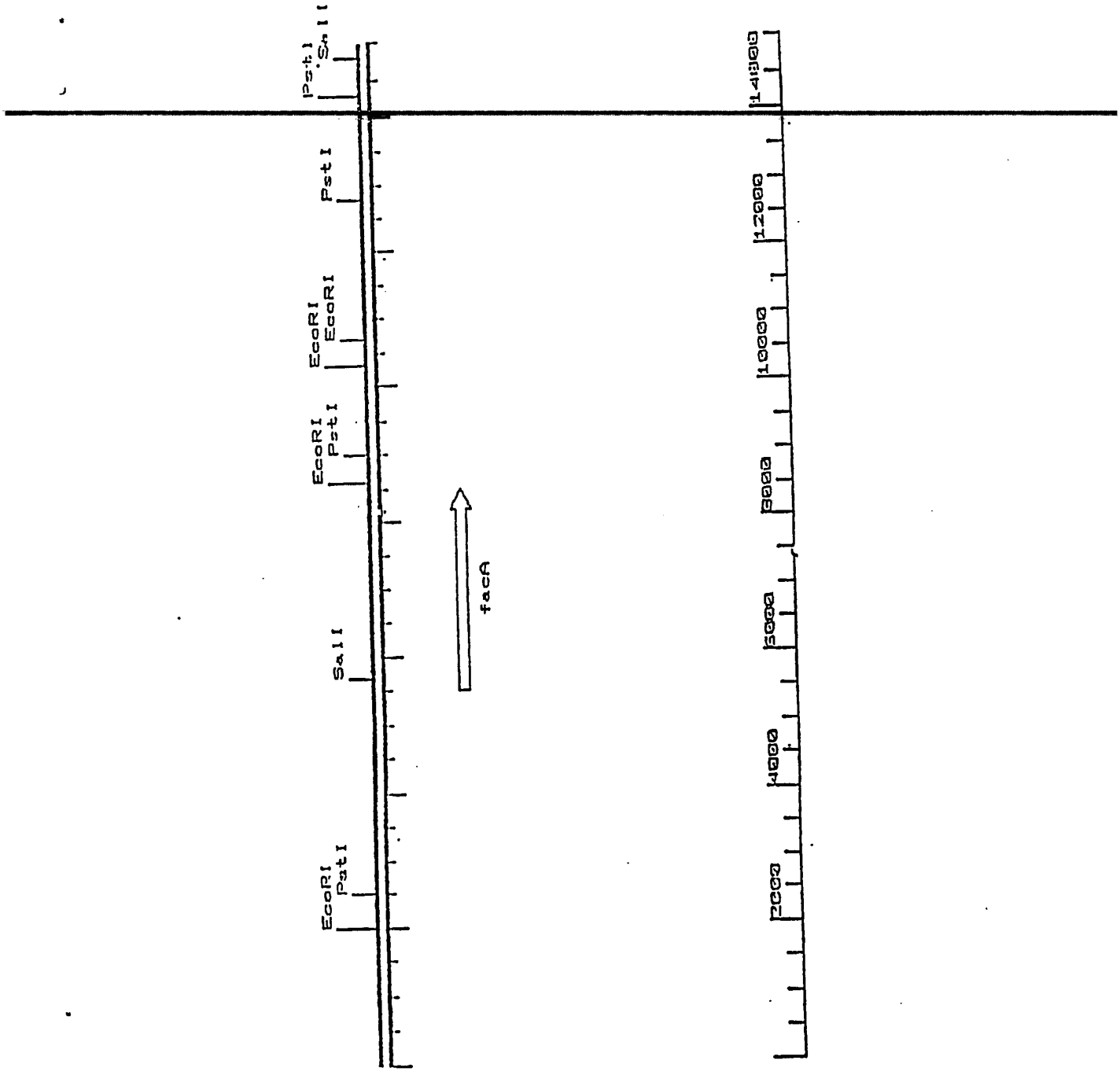
PstI
SalI
PstI
EcoRI
EcoRI
EcoRI
PstI
SalI
EcoRI
PstI
facA
14000
12000
10000
8000
6000
4000
2000

FIGURE 1

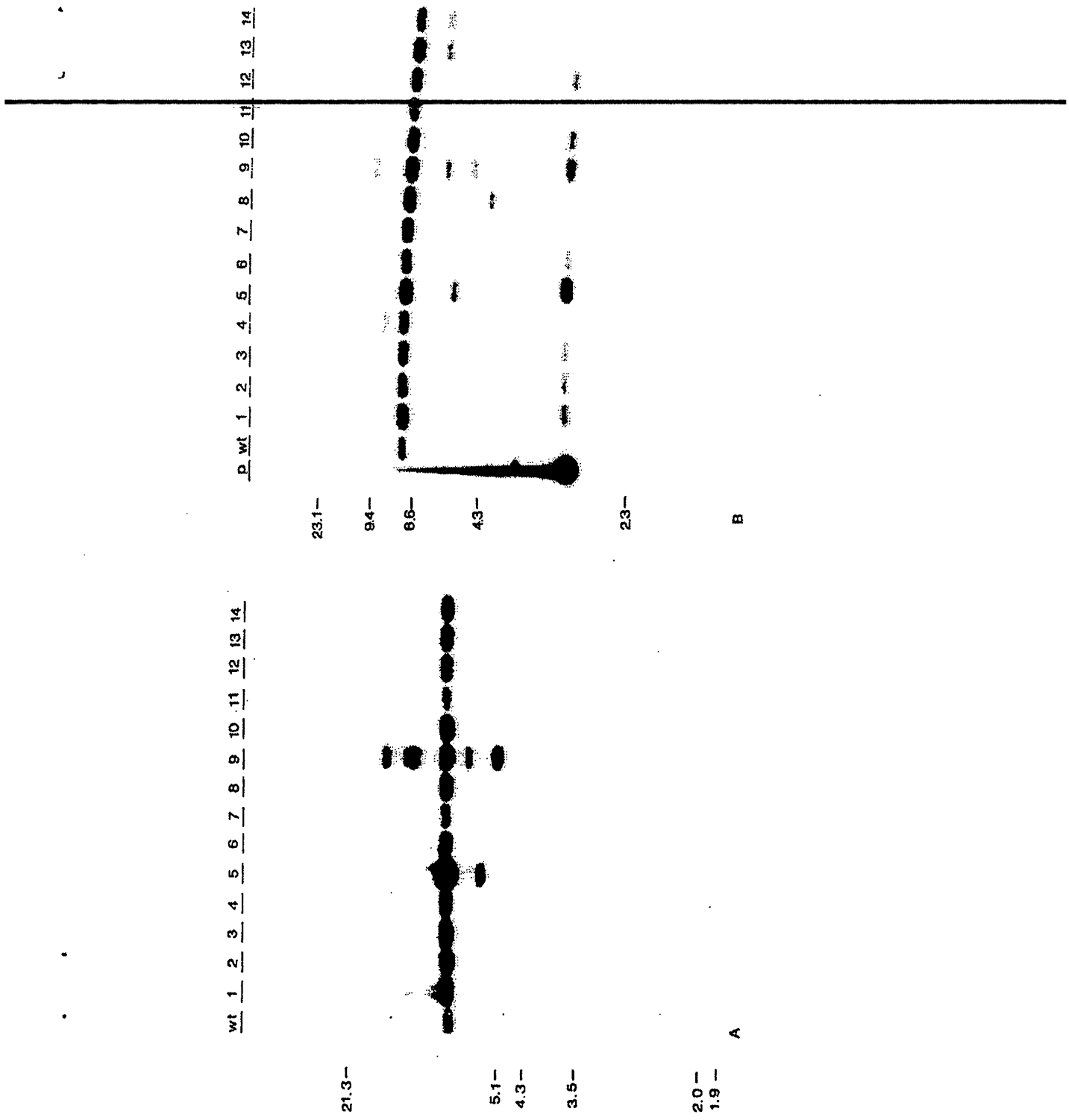
p wt 1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.1
9.4
6.6
4.3
2.3
B
wt 1 2 3 4 5 6 7 8 9 10 11 12 13 14
21.3
5.1
4.3
3.5
2.0
1.9
A

FIGURE 2

INTERNATIONAL SEARCH REPORT

PCT/NL 91/00203

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/80; C12P17/18; C12N15/52; C12N1/15
//(C12N1/15,C12R1:80)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	MOLECULAR MICROBIOLOGY vol. 4, no. 3, March 1990, OXFORD pages 451 - 460; I.F. CONNERTON ET AL: 'Comparison and cross-species expression of the acetyl-CoA synthetase genes of the ascomycete fungi, Aspergillus nidulans and Neurospora crassa' cited in the application	10-12, 14,15
Y	see the whole document	1-9

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 FEBRUARY 1992	10.03.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 113, no. 3, 16 July 1990, Columbus, Ohio, US; abstract no. 18877, C.A.CANTWELL ET AL: 'Cloning and expression of a hybrid Streptomyces clavuligerus cefE gene in Penicillium chrysogenum' page 172 ; see abstract & CURRENT GENETICS vol. 17, no. 3, 1990, pages 213 - 221; cited in the application	2-9
Y	JOURNAL OF GENERAL MICROBIOLOGY vol. 135, 1989, GREAT BRITAIN pages 2675 - 2678; J.A. HARGREAVES AND G. TURNER: 'Isolation of the acetyl-CoA synthetase gene from the corn smut pathogen Ustilago maydis' cited in the application see the whole document	1-9
Y	WO,A,9 010 074 (GLAXO GROUP LTD) 7 September 1990 see abstract	1-9
A	MOLECULAR AND GENERAL GENETICS vol. 218, 1989, BERLIN,DE pages 87 - 92; R.A. SANDEMAN AND M.J. HYNES: 'Isolation of the facA (acetyl-Coenzyme A synthetase) and acuE (malate synthase) genes of Aspergillus nidulans' cited in the application see the whole document	10-12, 14,15

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9100203 SA 52663

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/02/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9010074	07-09-90	EP-A- 0414870	06-03-91
		JP-T- 3504334	26-09-91